Planta (1997) 201: 293–297

Planta

Expression of the yeast *trehalose-6-phosphate synthase* gene in transgenic tobacco plants: pleiotropic phenotypes include drought tolerance

Carlos Romero, José M. Bellés, José L. Vayá, Ramón Serrano, Francisco A. Culiáñez-Macià

Instituto de Biología Molecular y Celular de Plantas, Universidad Politécnica de Valencia C.S.I.C. Camino de Vera s/n, E-46022 Valencia, Spain

Received: 8 July 1996 / Accepted: 10 October 1996

Abstract. The yeast *trehalose-6-phosphate synthase* gene (*TPS1*) was engineered under the control of the cauliflower mosaic virus regulatory sequences (CaMV35S) for expression in plants. Using *Agrobacterium*-mediated transfer, the gene was incorporated into the genomic DNA and constitutively expressed in *Nicotiana tabacum* L. plants. Trehalose was determined in the transformants, by anion-exchange chromatography coupled to pulsed amperometric detection. The non-reducing disaccharide accumulated up to 0.17 mg per g fresh weight in leaf extracts of transgenic plants. Trehalose-accumulating plants exhibited multiple phenotypic alterations, including stunted growth, lancet-shaped leaves, reduced sucrose content and improved drought tolerance. These pleiotropic effects, and the fact that water loss from detached leaves was not significantly affected by trehalose accumulation, suggest that synthesis of this sugar, rather than leading to an osmoprotectant effect, had altered sugar metabolism and regulatory pathways affecting plant development and stress tolerance.

Key words: Drought tolerance – *Nicotiana* – Osmotic stress – Transgenic plants – Trehalose

Introduction

Trehalose, a non-reducing disaccharide of glucose (α-D-glucopyranosyl α-D-glucopyranoside), accumulates in a large number of organisms in response to different stress conditions (Elbein 1974). In the yeast *Saccharomyces cerevisiae*, trehalose accumulation has been correlated with thermotolerance (De Virgilio et al. 1994) and resistance to cold and water stress (Mackenzie et al. 1988). Although the trehalose protective effect remains unclear at the molecular level, correlative evidence suggests that trehalose stabilises proteins and membrane structures under stress (Iwahashi et al.1995), a function probably resulting from the glass transition temperature, greater flexibility and chemical stability of trehalose compared to other sugars (Colaco et al. 1995). Trehalose is a rare sugar in higher vascular plants, where sucrose may act as the functional analogue (Hoekstra et al. 1992). Trehalose is present, however, in some desiccation-tolerant angiosperms (Drennan et al. 1993), and engineering trehalose accumulation into crop plants could improve drought and salinity tolerance (Serrano 1996). Here, we provide evidence that expression of the yeast *TPS1* or *CIF1* gene (Bell et al. 1992; González et al. 1992), encoding trehalose-6-phosphate synthase, is sufficient to determine trehalose accumulation in transgenic tobacco plants and that this accumulation alters the carbohydrate metabolism, induces morphological changes, and improves drought tolerance.

Material and methods

The *TPS1* coding region was isolated by polymerase chain reaction (PCR) from plasmid pMB14 (González et al. 1992), and cloned as a 1.5-kb *HindIII*/*Bam*HI fragment into plasmid pJIT 163 (Guerineau et al. 1992) creating pC1. The 2.8-kb *Kpn*I/*Xho*I DNA fragment from pC1, which contains the *TPS1* gene flanked by a cauliflower mosaic virus (CaMV) 35S promoter with a duplicated enhancer and by the CaMV polyadenylation sequence, was finally cloned into the binary plant vector pBin 19 (Bevan 1984) creating pC2. *Agrobacterium* helper strain LBA 4404 (Hoekema et al. 1983) was transformed with pC2 by high-voltage electroporation (Wen-jun and Forde 1989), and used for plant transformation. *Nicotiana tabacum* L. cv. SR1 (obtained from Dr. A. Spena. Max-Planck-Institut für Züchtungsforschung, Köln, Germany) leaf discs were agroinfected and the kanamycin-selected shoots transferred to MS rooting media (Murashige and Skoog 1962) and finally into soil in pots. Genomic DNA gel blots were performed using approximately 10 μg of DNA per track. Northern analysis of *TPS1* expression utilised approximately 5 μg of poly(A)$^{+}$RNA per track. Isolated DNA fragments were nick-translated in the presence of α-[^{32}P]dCTP to be used as probes (Maniatis et al. 1982). Filters were

Abbreviations: *TPS1*/*CIF1* = gene encoding trehalose-6-phosphate synthase; CaMV = cauliflower mosaic virus, CaMV35S = cauliflower mosaic virus regulatory sequences

Correspondence to: F.A. Culiáñez-Macià; Fax: 34(9)63877859; E-mail: faculia @ ibmcp.upv.es

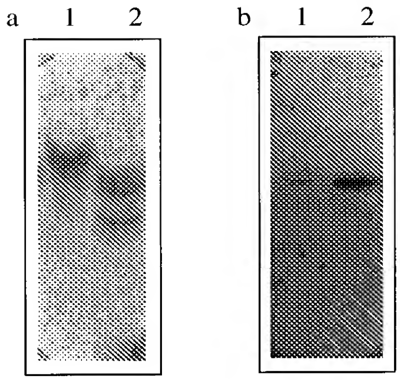

Fig. 1a,b. Correlation between copy number, mRNA amounts and altered phenotype in the incorporation and expression of the yeast *TPS1* gene in *Nicotiana tabacum* plants. **a** Southern blot of genomic DNA from two transgenic F_0 plants with low (*lane 1*) and high (*lane 2*) levels of morphological alterations. The DNA was digested with *Kpn*I, which cuts pC2 once at the 5′-35S end, and hybridised with radiolabelled *TPS1* DNA. **b** Northern blot of poly(A)$^+$RNA from the same plants as in **a** and probed with radiolabelled *TPS1* DNA. Radiolabelled tubulin DNA was used as a loading control

washed at high stringency (0.1 × SSC, 0.5% SDS at 65 °C i1 × SSC = 0.15 M NaCl, 0.015 M Na_3-citrate, pH 7). Extracts of soluble sugars were prepared as described by De Virgilio et al. (1994) from ≈5-cm-long young leaves (200 mg FW), harvested from the apex of 6-week-old soil-grown F_0 plants after Tarczynski et al. (1992). Separation of soluble carbohydrates was by high-performance anion-exchange chromatography coupled to pulsed amperometric detection (HPAE-PAD; Waters, Milford, Mass., USA), using a CarboPac PA1 column (250 mm long, 4 mm i.d., Dionex, Sunnyvale, Calif., USA) maintained at 22 °C (Rocklin and Pohl 1983). Quantitation of the sugars was by peak integration and comparison with sugar standards. To avoid mixing drought and starvation stress, for water stress 3-month-old F_1 transgenic tobacco plants, with and without the *TPS1* gene, were subjected to drought by withholding irrigation for 15 d. Leaves were considered dead, damaged or healthy when they were completely dry and brown, less than 50% yellow, or green, respectively. In all F_1 plant analyses, equally phenotypically altered lines were used.

Results and discussion

Using *Agrobacterium*-mediated transfer, the *TPS1* gene was stably integrated in the plant genome and expressed in the transgenic plants. In Southern blots, genomic DNA of transformed tobacco plants was probed with the yeast *TPS1* gene (Fig. 1) revealing prominent bands which corresponded in size to known restriction fragments of the *TPS1* gene construction. Transcripts of *TPS1* were detectable on Northern blots of leaf poly(A)$^+$RNA in the majority of *TPS1*-transformed plants. No bands were observed in control plants transformed with the binary vector pBin 19. The mRNA amounts correlated with the *TPS1* gene copy number and morphological alterations (Fig. 1). Those plants with high levels of morphological alterations had two gene copies (two bands in Fig. 1a) and increased mRNA amounts (Fig. 1b).

The carbohydrate profiles differed between control and transgenic F_0 plants (Fig. 2). Trehalose was present in the leaf extracts of transformants with levels of up to 0.17 mg·(g FW)$^{-1}$; (Table1). It could not be detected in control plants [< 0.02 mg·(g FW)$^{-1}$]. Trehalose accumulation was not dependent on the plant developmental stage since similar concentrations were found in leaves of younger or older F_1 plants. Decreases occurred in sucrose and glucose, and some unidentified peaks also

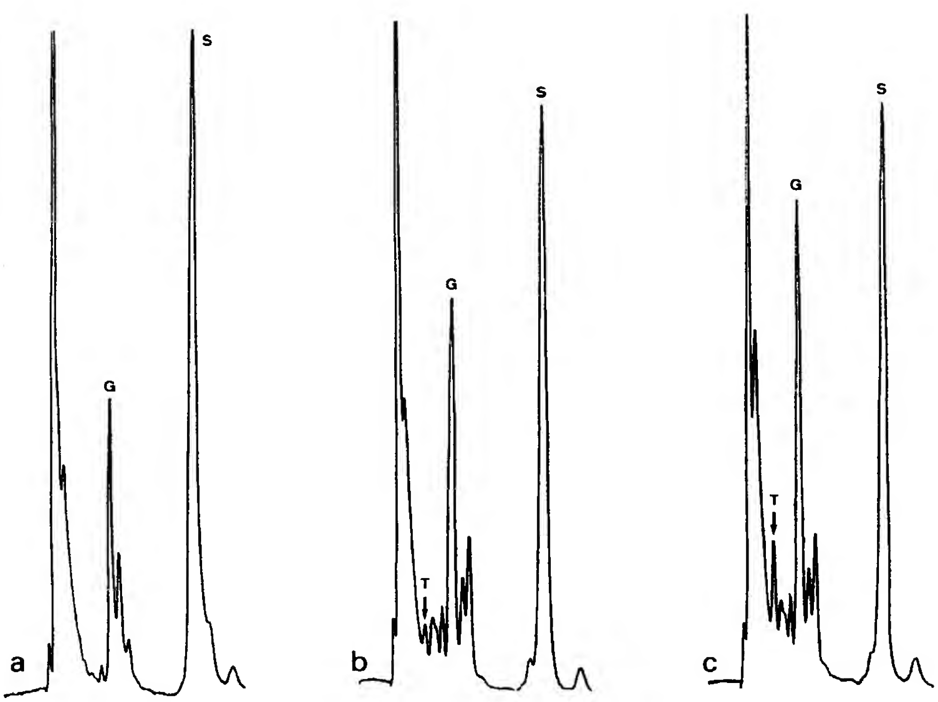

Fig. 2a-c. Carbohydrate profiles of control and transgenic tobacco plants. **a** Control F_0 plant transformed with the binary vector pBin 19. **b** transgenic F_0 plant transformed with pC2. **c** Extract of plants from **b** co-injected with 50 ng of trehalose. To better show the trehalose peak and avoid overhang of some peaks in the control-plant chromatogram, 3 µl of sugar extract was injected in **a** and 5 µl in **b,c**. *G*, glucose; *S*, sucrose; *T*, trehalose

Table 1. Sugar contents in leaves from control and *TPS1*-expressing plants. Data represent the mean value ± SD of eight plants [mg·(g FW)$^{-1}$]

	Control	*TPS1*
Sucrose	6.60 ± 1.70	4.00 ± 0.80
Glucose	0.16 ± 0.05	0.07 ± 0.02
Trehalose	n.d.[a]	0.15 ± 0.02

[a]Not detected [< 0.02 mg·(g FW)$^{-1}$]

underwent alterations in the transgenic plants. Work is in progress to identify these sugars. A large fraction (40%) of F_0 trehalose-accumulating plants exhibited different degrees of phenotypic change, related to loss of apical dominance, stunted growth, lancet-shaped leaves, and some sterility.

Water stress dramatically affected control tobacco plants, while wilting symptoms were attenuated in transgenic plants expressing the *TPS1* gene and were correlated with the degree of morphological change. Altered phenotype was always coupled with drought tolerance, although gene expression did not always perfectly correlate with trehalose accumulation, probably because of differences in trehalose activity. The F_1 plants segregated both the phenotypic alterations (Fig. 3a) and improved drought tolerance (Fig. 3b) observed in F_0 plants. Phenotypically altered F_1 plants segregated 1:3 from a single-copy low-morphologically altered F_0 line, and plants of the F_2 generation derived from these F_1 plants were homozygous and all showed an identical phenotype which was highly altered compared with the F_1 parental line. No phenotypic changes were observed in F_1 plants derived from F_0 control plants transformed with the binary vector pBin 19. Control plants exhibited dehydration and necrosis of leaves while F_1 phenotypically altered plants appeared to be much less affected (Fig. 4). Despite the protection observed in whole plants, there were no significant differences in the water loss of detached leaves of different sizes from control and transgenic F_0 plants (Fig. 5) or from F_1 seedlings, or young and adult F_1 plants.

The production of trehalose in the transgenic tobacco plants indicates that since trehalose formation is a two-step process, involving trehalose-6-phosphate synthase and trehalose-6-phosphate phosphatase, an endogenous phosphatase should be involved in this engineered biosynthesis in tobacco plants.

Focusing on the osmoprotectant role of trehalose in drought tolerance, it must be pointed out that the measured concentrations of this sugar in transgenic plants (< 0.5 mM) are too low, 5 to 10 times less than engineered mannitol and proline (Tarczynski et al. 1992; Kishor et al. 1995), for a conventional osmoprotectant effect. Trehalose accumulates to high concentrations in many micro-organisms under osmotic stress (> 0.1 M; Hottiger et al. 1987), but its accumulation in vascular plants is rare, probably because of reduced synthesis and also because it could be prevented by trehalase, an enzyme widely distributed in the plant kingdom (Müller et al. 1995). Thus, the relatively low trehalose concentration in desiccation-tolerant angiosperms (Drennan et al. 1993) and in the transgenic plants expressing the *TPS1* gene of the present work, suggests that trehalose may play a more complex role in preventing plant desiccation than just contributing to osmotic adjustment, a function that could be performed by other more abundant functional analogues such as sucrose, or other osmolites like proline and betaine. Our results are at odds with those of Holmström et al. (1996), since (i) we did not observe significant differences in the water loss of detached leaves of different sizes, as would be expected

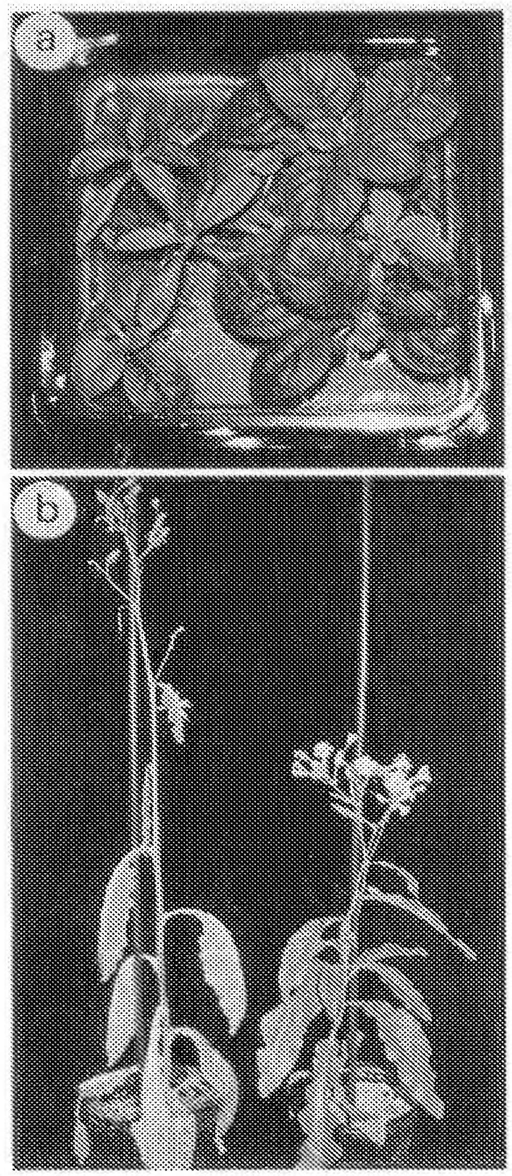

Fig. 3a,b. Segregation of both phenotypic changes and improved drought tolerance. **a** F_1 transgenic plants, from a single-copy *TPS1* gene F_0 plant, showing normal or morphologically altered lance-shaped leaves. **b** F_1 control plant (*left*), from an F_0 plant transformed with the binary vector pBin 19, and an F_1 phenotypically altered trehalose-accumulating transgenic plant (*right*), after 15 d of water stress

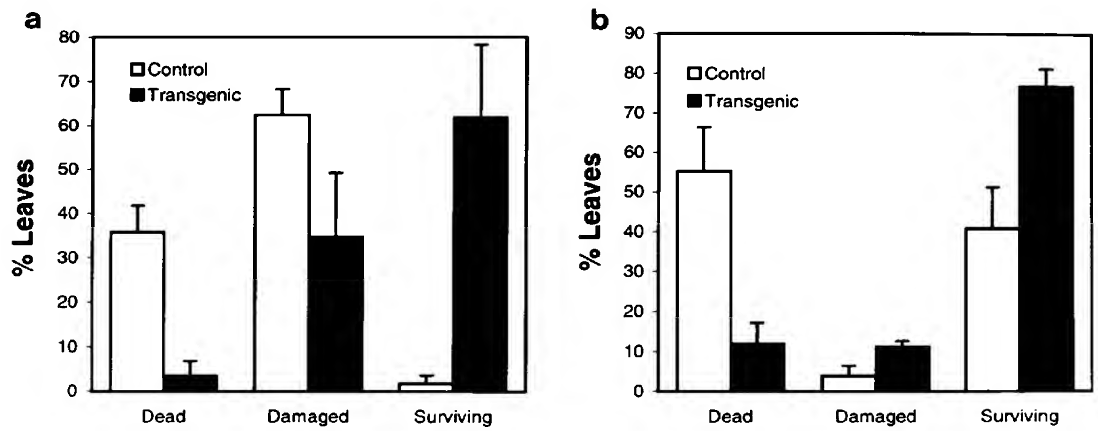

Fig. 4a,b. Comparison of the dehydration effect between F_1 control plants, derived from F_0 transformed with the binary vector pBin 19, and F_1 phenotypically altered trehalose-accumulating transgenic plants, expressed as the number of dead, damaged and surviving leaves after 15 d of water stress (**a**) or after 15 d of water stress followed by 15 d of re-watering (**b**). Each datum point corresponds to the mean value calculated from six plants of three different lines and the error bars correspond to the standard error

from an osmotic effect of trehalose, and (ii) we did observe that trehalose accumulation leads to phenotypic changes in tobacco. Similar phenotypic changes were also observed by Goddijn et al. (1995) who engineered trehalose-biosynthesis-expressing *E. coli otsA* and *otsB* genes in *Nicotiana tabacum.* Although all the plants constitutively expressing *TPS1* tolerated desiccation better than the controls, those plants showing severe morphological alterations had the highest tolerance under stress conditions.

The combination of these results indicates that the mechanism of the improved performance of trehalose-synthesising-plants in drought resistance is more subtle than a simple osmotic effect as in micro-organisms.

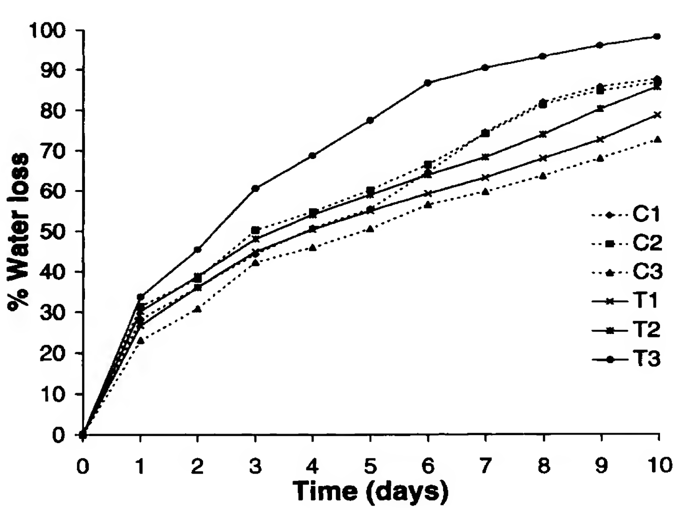

Fig. 5. Water loss of detached leaves from 4-month-old transgenic tobacco F_0 plants air-dried for 10 d at room temperature. T1, T2, and T3, transgenic *TPS1*-expressing plants. C1, C2, and C3, transgenic controls. Values are the average of leaves of different sizes

Trehalose has protein and membrane stabilisation properties superior to those of other sugars (Crowe et al. 1984; Colaco et al. 1995) but the low concentration accumulated in transgenic plants tempts us to speculate on the basis of the phenotypic effects observed, that rather than acting just as a compatible solute, it may affect metabolic and hormonal pathways leading to pleiotropic changes including stress-resistance. The carbohydrate profile differed between control and transgenic plants, suggesting changes in carbohydrate metabolism. Recently, one research team emphasised that the control of gene expression plays a fundamental role in carbohydrate-mediated feedback or sink-regulated inhibition of photosynthesis, linking this sugar regulation mechanism to a switch that facilitates the cell defence response against a variety of stimuli (Jang and Sheen 1994). Furthermore, systemic acquired resistance mediated by the ectopic expression of yeast invertase has been related to a possible hexose sensing in the secretory pathway (Herbers et al. 1996). Trehalose could have some ability to cause repression, which would be dependent on its hydrolysis to glucose by plant trehalase, as was suggested for sucrose repression activity, which is presumably dependent on its hydrolysis to glucose and fructose by invertase (Goldschmidt and Huber 1992). It has previously been reported that there is a correlation between trehalose accumulation in plants and interference in sugar metabolism fall in sucrose content, inhibition of synthesis of cell wall polysaccharides and potential toxicity (Veluthambi et al. 1982). Thus, trehalose could act, as a direct or indirect signal, in similar sugar-sensing and signalling pathways controlling plant gene expression. An alternative explanation for the effects of *TPS1* expression in higher plants is, as has been proposed in yeast (Thevelein 1994), that the activity of trehalose phosphate synthase reduces the concentration of sugar phosphates, and that these metabolites are signalling molecules for different transduction pathways affecting plant development and stress tolerance. Work is in progress to check if the morphological and carbohydrate alterations produced by expression of the *TPS1* gene are possibly linked with the plant stress-responsive pathway. Further physiological research needs to be done before trehalose biosynthesis can be successfully engineered in a wider range of crop

plants, thus increasing their resistance to environmental stress. Molecular analyses of these phenotypically altered plants are presently under way in order to obtain a clearer picture about the relations between trehalose and drought tolerance.

We gratefully acknowledge the support provided by Dr. M. Pagès (CID-CSIC. Barcelona, Spain) and the donation of plasmids pMB14 by Dr. C. Gancedo (IIB-CSIC. Madrid, Spain) and pJIT163 by Dr. P. Mullineaux (John Innes Centre. Norwich, UK). This material is based upon work supported under a Consellería d'Agricultura Generalitat Valenciana Research Fellowship to CR and by the I+D-CICYT Research Project BIO96-1196-C02-02 from the M.E.C. of Spain.

References

Bell W, Klaassen P, Ohnacker M. Boller T, Herweijer M, Schoppink P, Van der Zee P, Wiemken A (1992) Characterization of the 56-kDa subunit of yeast trehalose-6-phosphate synthase and cloning of its gene reveal its identity with the product of *CIFI*, a regulator of carbon catabolite inactivation. Eur J Biochem 209: 951–959

Bevan M (1984) Binary *Agrobacterium* vectors for plants transformation. Nucleic Acids Res 12: 8711–8721

Colaco C, Kampinga J, Roser B (1995) Amorphous stability and trehalose. Science 268: 788–788

Crowe J, Crowe L, Chapman D (1984) Preservation of membranes in anhydrobiotic organism: the role of trehalose. Science 223: 701–703

De Virgilio C, Hottiger T, Dominguez J, Boller T, Wiemken A (1994) The role of trehalose synthesis for the acquisition of thermotolerance in yeast I. Genetic evidence that trehalose is a thermoprotectant. Eur J Biochem 219: 179–186

Drennan PM, Smith MT, Goldsworthy D, Van Staden J (1993) The occurrence of trehalose in the leaves of the desiccation-tolerant angiosperm *Myrothamnus flabellifolius* Welw. J Plant Physiol 142: 493–496

Elbein A (1974) The metabolism of α,α-trehalose. Adv Carbohyd Chem Bi 30: 227–256

Goddijn OJM, Verwoerd TC, Voogd E, Krutwagen R, Degraaf P, Vandun K, Delaat A, Vandenelzen P, Damm B, Pen J (1995) Transgenic tobacco plants as a model-system for the production of trehalose. Plant Physiol 108: S786

Goldschmidt EE, Huber SC (1992) Regulation of photosynthesis by end-product accumulation in leaves of plant storing starch, sucrose, and hexose sugars. Plant Physiol 99: 1443–1448

González MI, Strucka R, Blázquez MA, Feldmann H, Gancedo C (1992) Molecular cloning of *CIFI*, a yeast gene necessary for growth on glucose. Yeast 8: 183–192

Guerineau F, Lucy A, Mullineaux P (1992) Effect of two consensus sequences preceding the translation initiator codon on gene expression in plant protoplasts. Plant Mol Biol 18: 815–818

Herbers K, Meuwly P, Frommer WB, Métraux JP, Sonnewald U (1996) Systemic acquired resistance mediated by the ectopic expression of invertase: possible hexose sensing in the secretory pathway. Plant Cell 8: 793–803

Hoekema A, Hirsch PR, Hooykaas PJJ, Schilperoort RA (1983) A binary plant vector strategy based on separation of *vir*- and T-region of the *Agrobacterium tumefaciens* Ti plasmid. Nature 303: 179–180

Hoekstra FA, Crowe LM, Van Rockel T, Vermeer E (1992) Do phospholipids and sucrose determine membrane phase transitions in dehydrating pollen species? Plant Cell Environ 15: 601–606

Holmström K-O, Mäntylä E, Welin B, Mandal A, Palva ET, Tunnela OE, Londesborough J (1996) Drought tolerance in tobacco. Nature 379: 683–684

Hottiger T, Boller T, Wiemken A (1987) Rapid changes of heat and desiccation tolerance correlated with changes of trehalose content in *Saccharomyces cerevisiae*. FEBS Lett 255: 5518–5522

Iwahashi H, Obuchi K, Fujii S, Komatsu Y (1995) The correlative evidence suggesting that trehalose stabilizes membrane-structure in the yeast *Saccharomyces cerevisiae*. Cell Mol Biol 41: 763–769

Jang J-C, Sheen J (1994) Sugar sensing in higher plants. Plant Cell 6: 1665–1679

Kishor PBK, Hong Z, Miao G-H, Hu C-AA, Verma DPS (1995) Overexpression of Δ^1-pyrroline-5-carboxylate synthetase increases proline production and confers osmotolerance in transgenic plants. Plant Physiol 108: 1387–1394

Mackenzie KF, Singh KK, Brown AD (1988) Water stress plating hypersensitivity of yeast: protective role of trehalose in *Saccharomyces cerevisiae.* J Gen Microbiol 134: 1661–1666

Maniatis T, Fritsch EF, Sambrook J (1982) Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY

Müller J, Boller T, Wiemken A (1995) Trehalose and trehalase in plants: recent developments. Plant Sci 112: 1–9

Murashige T, Skoog F (1962) A revised medium for rapid growth and bioassay with tobacco tissue cultures. Physiol Plant. 15: 473–497

Rocklin RD, Pohl CA (1983) Determination of carbohydrates by anion exchange chromatography with pulsed amperometric detection. J Liq Chromatogr 6: 1577–1590

Serrano R (1996) Salt tolerance in plants and microorganism: toxicity, targets and defense responses. Int Rev Cytol 165: 1–52

Tarczynski MC, Jensen RG, Bohnert HJ (1992) Expression of a bacterial *mtlD* gene in transgenic tobacco leads to production and accumulation of mannitol. Proc Natl Acad Sci USA 89: 2600–2604

Thevelein JM (1994) Signal transduction in yeast. Yeast 10: 1753–1790

Veluthambi K, Mahadevan S, Maheshwari R (1982) Trehalose toxicity in *Cuscuta reflexa*: cell wall synthesis is inhibited upon trehalose feeding. Plant Physiol 70: 686–688

Wen-jun S, Forde BG (1989) Efficient transformation of *Agrobacterium spp.* by high voltage electroporation. Nucleic Acids Res 17: 8385–8385